Deficiency in *SLC25A1*, Encoding the Mitochondrial Citrate Carrier, Causes Combined D-2- and L-2-Hydroxyglutaric Aciduria

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The Krebs cycle is of fundamental importance for the generation of the energetic and molecular needs of both prokaryotic and eukaryotic cells. Both enantiomers of metabolite 2-hydroxyglutarate are directly linked to this pivotal biochemical pathway and are found elevated not only in several cancers, but also in different variants of the neurometabolic disease 2-hydroxyglutaric aciduria. Recently we showed that cancer-associated *IDH2* germline mutations cause one variant of 2-hydroxyglutaric aciduria. Complementary to these findings, we now report recessive mutations in *SLC25A1*, the mitochondrial citrate carrier, in 12 out of 12 individuals with combined D-2- and L-2-hydroxyglutaric aciduria. Impaired mitochondrial citrate efflux, demonstrated by stable isotope labeling experiments and the absence of SLC25A1 in fibroblasts harboring certain mutations, suggest that *SLC25A1* deficiency is pathogenic. Our results identify defects in *SLC25A1* as a cause of combined D-2- and L-2-hydroxyglutaric aciduria.

D-2-hydroxyglutarate (D-2-HG) and L-2-hydroxyglutarate (L-2-HG) are associated with the Krebs (tricarboxylic acid/citrate) cycle,[1] with rare disorders,[2] and with certain types of cancer.[3–5] D-2-hydroxyglutaric aciduria (D-2-HGA), characterized by elevated levels of D-2-HG, is either autosomal recessive, resulting from mutations in *D2HGDH*[6] (type I [MIM 600721]), or is caused by recurrent usually de novo dominant gain-of-function mutations in *IDH2*[7] (type II [MIM 613657]). Manifestations of both types of D-2-HGA include developmental delay, hypotonia, and seizures.[2] Recessive mutations in *L2HGDH* result in L-2-hydroxyglutaric aciduria[8,9] (L-2-HGA [MIM 236792]), and predominant signs include macrocephaly, developmental delay, epilepsy, and cerebellar ataxia. Combined D-2- and L-2-hydroxyglutaric aciduria (D,L-2-HGA), characterized by elevated levels of both D-2-HG and L-2-HG in body fluids, is not caused by mutations in any of the above-mentioned genes and mainly manifests in a severe neonatal epileptic encephalopathy, absence of developmental progress, and often early death[10] (Table S1 available online).

Elevated D-2-HG levels are also found in gliomas and myeloid leukemias[3,11] and are associated with recurrent mutations in the isocitrate dehydrogenase (IDH) genes *IDH1* or *IDH2*. These mutations disable the normal ability of IDH to convert isocitrate to α-ketoglutarate and result in a new function: converting α-ketoglutarate to D-2-HG.[3] However, elevated D-2-HG and L-2-HG levels are also found in thyroid carcinomas without *IDH* mutations,[4] caused by still unknown mechanisms. The exact role of "oncometabolite" 2-hydroxyglutarate (2-HG) in cancer is not yet fully understood, but recent studies indicate an important role in epigenetics.[12–14] Identifying the genetic cause of combined D,L-2-HGA could therefore not only improve our knowledge of the pathophysiology of this neurometabolic disorder, but also provide further insight into its role in cancer.

We performed whole-exome sequencing to identify the genetic basis of combined D,L-2-HGA in DNA of three affected individuals and the unaffected parents of one of them (subject 1). A total of 13 affected individuals

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Table 1. Homozygous or Compound Heterozygous _SLC25A1_ Mutations Found in Combined D,L-2-HGA Cases

Subject	Nucleotide Change	Deduced Effect	Exon	SIFT Score[a]
1[b]	c.578C>G	p.Ser193Trp	6	0.00
2[b]	c.844C>G	p.Arg282Gly	9	0.00
3[c]	c.844C>T	p.Arg282Cys	9	0.00
4[d]	c.821C>T	r.820_821del (p.Ala274Ilefs*24)	8	–
5	c.18_24dup	p.Ala9Profs*82	1	–
	c.499G>A	p.Gly167Arg	5	0.00
6	c.18_24dup	p.Ala9Profs*82	1	–
	c.134C>T	p.Pro45Leu	2	0.00
7[b]	c.18_24dup	p.Ala9Profs*82	1	–
	c.768C>G	p.Tyr256*	8	–
8[b]	c.430G>C	p.Glu144Gln	4	0.00
9	c.18_24dup	p.Ala9Profs*82	1	–
10[b]	c.605T>C	p.Met202Thr	6	0.01
	c.890A>G	p.Tyr297Cys	9	0.00
11	c.821C>T	r.820_821del (p.Ala274Ilefs*24)	8	–
12[b]	c.517_526del	p.Arg173Glyfs*2	5	–
	c.821C>T	r.820_821del (p.Ala274Ilefs*24)	8	–

The primers used for PCR amplification and sequencing are summarized in Table S3. _SLC25A1_ RefSeq NM_005984.2.
[a]All missense mutations were predicted with SIFT to be damaging (p < 0.05).
[b]Homozygosity or compound heterozygosity was confirmed by DNA sequencing of the parents.
[c]Homozygous mutation was also found with DNA sequencing in affected sibling.
[d]Mutation was confirmed in mRNA isolated from fibroblasts.

diagnosed with combined D,L-2-HGA from 12 unrelated families (Arabic, European, or Latin-American descent) were included in this study. They all showed elevated levels of D-2-HG and L-2-HG in urine (Table S2). Written consent was obtained from participants and/or their guardians for the exome sequencing analysis; for all other participants informed consent was waived because of the retrospective nature of the study. The study received Institutional Review Board approval from the VU University Medical Center Amsterdam (registered with the US Office of Human Research Protections under number IORG0002436). Genomic DNA was extracted from blood, and 3 μg was fragmented via Covaris technology. Fragments were ligated with adaptors (Illumina), amplified by ligation-mediated PCR, and enriched with SureSelect Human All Exon v.2 Kit (Agilent). Sequencing was performed on a HiSeq2000 (Illumina) and generated paired-end 100 bp reads. Mapping of the reads to the human reference genome (UCSC Genome Browser hg19) was done with Burrows-Wheeler Aligner.[15] VarScan[16] software was used for calling SNPs and indels. Annotation of the obtained

variants was performed with ANNOVAR.[17] We obtained a mean coverage depth of the exomes ranging from 83× to 109× and searched for nonsynonymous single-nucleotide variants (SNVs) in the family of subject 1, not present in dbSNP (132) or 1000 Genomes, and with a SIFT[18] prediction score < 0.05. Assuming a model in which mutations have a recessive effect,[10] we identified nonsynonymous SNVs in three genes (_OR52K1_, _OR51I2_, and _SLC25A1_ [RefSeq accession number NM_005984.2, MIM 190315]) that were homozygous (100% of the reads) in the affected individual and heterozygous (50% of the reads) in either parent with >40× coverage depth. Of these three genes, only SNVs in _SLC25A1_, with the above-mentioned criteria, were identified in the other two affected individuals (subjects 2 and 3). A similar search for small insertions or deletions did not identify any additional genes. Sanger sequencing of the ORF of _SLC25A1_ confirmed the mutations in these three individuals and their families and revealed mutations in nine additional unrelated affected subjects (Table 1). In total, 12 different mutations were detected (Figure 1A): eight missense, one nonsense, two frameshift (of which one was recurrent in four unrelated individuals), and one mutation resulting in a splicing error. The latter mutation, c.821C>T, found in three unrelated individuals, is located at the most 3′ end of exon 8 and introduces a splice donor site, resulting in a deletion in mRNA (r.820_821del), and is predicted to cause a frameshift at the protein level (p.Ala274Ilefs*24). Sequencing of mRNA from fibroblasts derived from subject 4 confirmed a 2 bp deletion (Figure S2). All missense mutations were predicted with SIFT to be damaging (Table 1).

With immunoblot analysis, we examined SLC25A1 in fibroblasts available from seven affected individuals (Figures 1B and S3). SLC25A1 was detected in cells from control subjects and all subjects harboring homozygous or compound heterozygous missense mutations. However, in cells from subjects containing two alleles with truncating mutations (subjects 7 and 9), no protein was detected, probably as a consequence of nonsense-mediated decay. The homozygous splice site mutation c.821C>T (subject 4; p.Ala274Ilefs*24) had reduced levels of SLC25A1. This could be explained by some authentic spliced protein product (p.Ala274Val), or alternatively, because the frameshift occurs at the C-terminal end, this protein could still be immunodetected but is degraded at higher rate.

The location of _SLC25A1_ within the human genome was previously described to be involved in other disorders.[19] _SLC25A1_ is located on chromosome 22q11 within the critical region deleted in DiGeorge syndrome[20] (MIM 188400). DiGeorge syndrome is a rather common congenital disorder (prevalence of 1:4,000) caused by a hemizygous deletion of chromosome 22q11.2 (containing ~45 genes), with a wide variety of clinical abnormalities including heart defects, velopharyngeal inadequacies, recurrent infections, and learning disabilities.[21] Consequently, individuals with DiGeorge syndrome are heterozygous carriers of SLC25A1 deficiency.
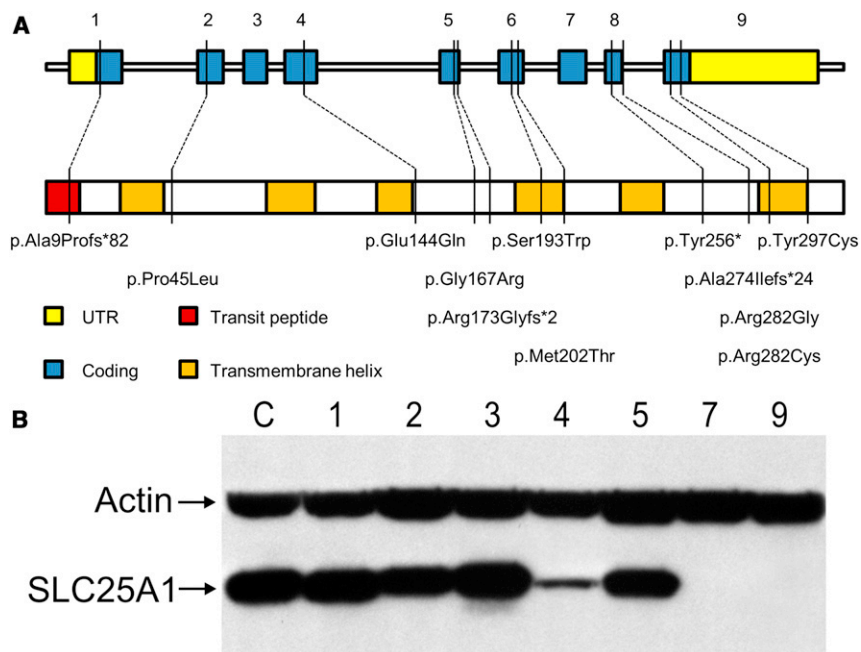
Figure 1. Schematic Overview of Genomic Organization and Protein Domain Structure of SLC25A1

(A) *SLC25A1* contains nine exons that encode a 311 amino acid long product. The first 13 amino acids are a transit peptide (red) for mitochondrial targeting, and the mature protein contains six membrane-spanning helices (orange). Locations of the 12 different identified mutations are shown. An alignment of the involved amino acids with different species is shown in Figure S1.

(B) Immunoblot analysis of SLC25A1 accumulation in fibroblasts derived from control "C" and affected subjects 1–5, 7, and 9. SLC25A1 is immunodetected in control, subjects 1–3 (homozygous missense mutations), and subject 5 with mutation c.18_24dup (p.Ala9Profs*82) and c.499G>A (p.Gly167Arg). Low detection in subject 4 (homozygous mutation c.821C>T [p.Ala274Ilefs*24]) and no detection in subject 7 with mutation c.18_24dup (p.Ala9Profs*82) and c.768C>G (p.Tyr256*) and subject 9 (homozygous mutation c.18_24dup [p.Ala9Profs*82]) is consistent with the presence of truncating mutations. The whole-blot image and a blot with a lower exposure time are shown in Figure S3.

Expression of *SLC25A1* mRNA is high in liver, kidney, and pancreas.[22] It encodes a protein of 311 amino acids containing six transmembrane helices that is located in the inner mitochondrial membrane. SLC25A1 mediates the efflux of mitochondrial citrate or isocitrate in exchange for cytosolic malate.[23] Citrate and isocitrate are intermediates of the Krebs cycle, which occurs in the matrix of mitochondria and is responsible for energy production. Citrate exported by SLC25A1 out of the mitochondria into the cytosol is converted into acetyl coenzyme A (acetyl-CoA), which is essential for fatty acid and sterol synthesis.[24] Furthermore, cytosolic citrate is an important regulator of glycolysis, because it is an allosteric inhibitor of 6-phosphofructokinase (PFK1), the most important regulator enzyme of glycolysis.[24]

To examine the effect of SLC25A1 deficiency, we retrospectively measured the urinary citrate and isocitrate levels of individuals with combined D,L-2-HGA by using liquid chromatography-tandem mass spectrometry. Compared to age-matched controls (n = 40), significantly lower levels of both metabolites were detected (Figures 2A and 2B; Table S2). Interestingly, the eldest affected individual (subject 10) in our cohort showed the highest levels of citrate and isocitrate (Table S2). To further study SLC25A1 activity, we incubated combined D,L-2-HGA-affected fibroblasts (n = 7) and controls (n = 2) with [U-$^{13}C_6$]glucose-enriched medium. [U-$^{13}C_6$]glucose is metabolized via glycolysis and enters the Krebs cycle predominantly as [$^{13}C_2$]acetyl-CoA. The condensation reaction of [$^{13}C_2$]acetyl-CoA and oxaloacetate results in intramitochondrial [$^{13}C_2$]citrate. In combined D,L-2-HGA-affected fibroblasts, we detected lower [$^{13}C_2$]citrate levels in the culture medium than in controls (Figure 2C; Table S2). This demonstrates that *SLC25A1* mutations result in impaired citrate efflux. Interestingly, fibroblasts from subject 7 (c.18_24dup and c.768C>G) and subject 9 (c.18_24dup) showed ~7% efflux as compared to controls, indicating that citrate can leave to a lesser extent the mitochondrial matrix via other means than SLC25A1 or is formed in the cytosol.

Individuals with combined D,L-2-HGA excrete increased levels of several Krebs cycle intermediates including α-ketoglutarate, malate, fumarate, and succinate, accompanied with decreased levels of citrate and isocitrate (Table S2). We speculate that the increased urinary excretion of D-2-HG and L-2-HG is caused by the inability of mitochondria to export citrate and isocitrate into the cytosol. This would probably result in increased mitochondrial concentrations, and subsequently higher excretion, of Krebs cycle metabolites downstream of isocitrate. We therefore measured the above-mentioned metabolites in homogenates of fibroblasts from controls and affected individuals (Table S4). Compared to controls, the levels of D-2-HG and L-2-HG in affected individuals are increased, which is in agreement with previously reported data.[25] Additionally, citrate levels were lower in affected fibroblasts, but isocitrate and the other metabolites were normal compared to controls.

In conclusion, *SLC25A1* mutations cause combined D,L-2-HGA as well as altered levels of several Krebs cycle intermediates. This finding provides the basis for an understanding of the pathophysiology and future therapeutic research concerning this disease that may include citrate
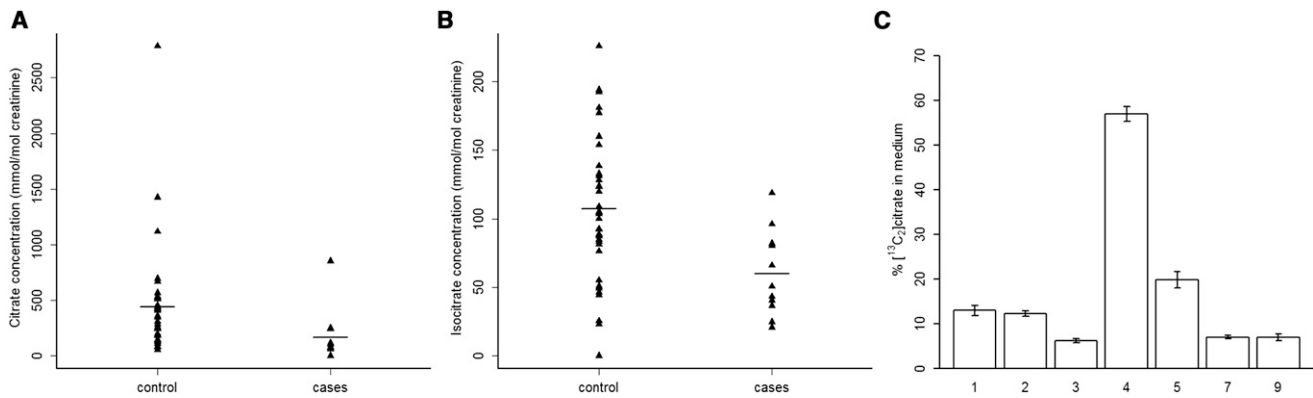
Figure 2. Decreased Citrate and Isocitrate Levels in Combined D,L-2-HGA

(A and B) Urinary levels of (A) citrate and (B) isocitrate were measured with LC-MS/MS in samples of controls (n = 40) and of individuals with combined D,L-2-HGA (n = 11) with *SLC25A1* mutations. Horizontal lines indicate the group sample means. The differences observed between controls and combined D,L-2-HGA-affected individuals were statistically significant by Mann-Whitney's test for both citrate (p = 0.0011) and isocitrate (p = 0.0015) levels (p < 0.05 was considered significant). Urinary levels of an unrelated metabolite (α-aminoadipic acid) was not significantly different between individuals with combined D,L-2-HGA and controls (Figure S4).

(C) Fibroblast cultures derived from affected subjects (n = 7) were given [U-^{13}C$_6$]glucose, which is metabolized by glycolysis predominantly to [^{13}C$_2$]acetyl-CoA. [^{13}C$_2$]acetyl-CoA is converted to [^{13}C$_2$]citrate within mitochondria via the Krebs cycle. Levels of [^{13}C$_2$]citrate were measured after 72 hr incubation in culture medium and are expressed as percentage compared to control fibroblasts (n = 2). Error bars indicate standard error of mean of replicate measurements (n = 3).

supplementation. SLC25A1 deficiency may be underdiagnosed and inclusion in the differential diagnosis of mitochondrial disorders should be considered. Further research is needed to unravel how SLC25A1 defects cause elevation of 2-HG, a cancer-associated metabolite. Although no cancer was diagnosed in our combined D,L-2HGA cohort (Table S1), an important role for citrate or SLC25A1 has previously been implied in epigenetics or cancer biology.[26–30] The absence of cancer cases in our combined D,L-2-HGA cohort might be explained by the early age of death. Another explanation might be that the L-2-HG levels in our cohort are not as high as in L-2-HGA-affected individuals. Only L-2-HGA has previously been associated with higher incidences of cancer.[5] In both types of D-2-HGA, no higher incidence of cancer has been found.[2] Moreover, certain cancer types show elevated levels of 2-HG but contain no mutations in *IDH1* or *IDH2*.[4] It would therefore be interesting to further investigate the role of citrate and SLC25A1 in these cancer types.

Supplemental Data

Supplemental Data include four figures and four tables and can be found with this article online at http://www.cell.com/AJHG/.

Acknowledgments

Web Resources

The URLs for data presented herein are as follows:

1000 Genomes, http://browser.1000genomes.org
dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org/
SIFT, http://sift.bii.a-star.edu.sg/
UCSC Genome Browser, http://genome.ucsc.edu

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